BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

As rescanning documents *will not* correct images, please do not report the images to the Image Problem Mailbox.

PCT

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : A61K 31/56, 31/715, C08B 37/16 — A1

(11) International Publication Number: WO 89/ 06536

(43) International Publication Date: 27 July 1989 (27.07.89)

(21) International Application Number: PCT/US89/00175

(22) International Filing Date: 17 January 1989 (17.01.89)

(31) Priority Application Numbers: 145,407; 295,633

(32) Priority Dates: 19 January 1988 (19.01.88); 10 January 1989 (10.01.89)

(33) Priority Country: US

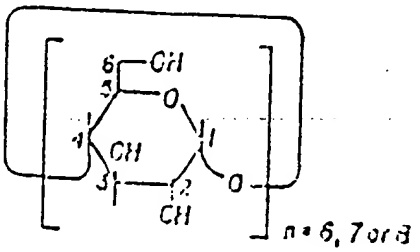
(71)(72) Applicants and Inventors: FOLKMAN, Moses, Judah [US/US]; 18 Chatham Circle, Brookline, MA 02146 (US). WEISZ, Paul, Burg [US/US]; 3 Delaware Rim Drive, Yardley, PA 19067 (US).

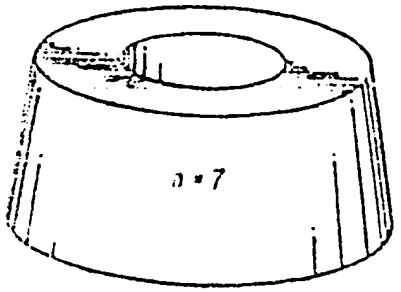
(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155 Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB, GB (European patent), IT (European patent), JP, KR, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: GROWTH INHIBITING AGENT AND THE USE THEREOF





(57) Abstract

Pathological or otherwise undesirable cell or tissue growth in mammals, including humans, is inhibited by administering thereto a composition exemplified by (1) a water-soluble cyclodextrin sulfate salt, together with (2) a growth-inhibiting organic compound. The growth-inhibiting compound (2) may be a steroid having no inhibiting effect in the absence of (1), or an organic compound which may be an active growth inhibitor, the action of which is potentiated by (1). The invention provides a method for inhibiting angiogeneses and controlling the growth of tumors as well as treating other diseases and pathological conditions associated with undesired cell or tissue growth, including angiogenesis.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	ML	Mali
AU	Australia	GA	Gabon	MR	Mauritania
BB	Barbados	GB	United Kingdom	MW	Malawi
BE	Belgium	HU	Hungary	NL	Netherlands
BG	Bulgaria	IT	Italy	NO	Norway
BJ	Benin	JP	Japan	RO	Romania
BR	Brazil	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic	KR	Republic of Korea	SE	Sweden
CG	Congo	LI	Liechtenstein	SN	Senegal
CH	Switzerland	LK	Sri Lanka	SU	Soviet Union
CM	Cameroon	LU	Luxembourg	TD	Chad
DE	Germany, Federal Republic of	MC	Monaco	TG	Togo
DK	Denmark	MG	Madagascar	US	United States of America
FI	Finland				

GROWTH INHIBITING AGENT AND THE USE THEREOF

1. FIELD OF THE INVENTION

This invention is concerned broadly with the inhibition of pathological or undesired cell or tissue growth in mammals by use of a new growth-inhibiting composition. More specifically, the present invention is directed to a growth-inhibiting composition comprising a highly soluble cyclodextrin derivative and a latent or active growth-inhibiting compound, and to the use of this composition to inhibit undesired or pathological growth, including angiogenesis which is asscciated with, inter alia, the growth of malignant tumors.

2. BACKGROUND OF THE INVENTION

2.1. HEPARIN AND INHIBITION OF ANGIOGENESIS

Angiogenesis, the induction of growth of new capillary blood vessels, is important in normal processes such as development of the embryo, formation of the corpus luteum and healing of wounds. It is also an important component in pathological processes such as chronic inflammation, certain immune responses, and neoplasia. It is now accepted that angiogenesis is induced by most malignant tumors and that it is necessary for their continued growth and survival. It is also recognized that angiogenesis is a major component of a number of ophthamological pathologies including such as diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma. Additionally, angiogenesis is now recognized as a major component in other non-neoplastic pathological conditions including rheumatoid arthritis, in which abnormal capillary growth can destroy joint cartilage; hemanogiomas, in which abnormal capillary proliferation appears in newborns and can persist for up to 2 years; angiofibromas which develop in the nasopharynx; and psoriasis, in which excessive proliferation and shedding may be dependent on abnormal

capillary growth in the dermis [see Folkman and Klagsbrun, Science 235:442 (1987)].

It has been previously discovered that heparin (or heparin fragments) and cortisone will co-act together to inhibit angiogenesis. This is described in U.S. Patent Application Serial No. 641,305 filed August 15, 1984, the contents of which are incorporated herein by reference. When administered together to mice with certain kinds of tumors, this combination can inhibit the generation of essential capillary vessels that support tumor growth, and can cause the collapse of the blood supply which supports the tumors. A review of the history of this discovery and of related subject matter is contained in the publication "How is Blood Vessel Growth Regulated in Normal and Neoplastic Tissue?" (G.H.A. Clowes Memorial Award Lecture), Judah Folkman, Cancer Research, 46:467 (1986) the contents of which are incorporated herein by reference for background.

Cortisone is an anti-inflammatory agent that by itself does not have the ability to inhibit capillary growth. It has been reported in Shubik et al., J. Nat'l Cancer Inst. 57:769 (1976) that 6 a-methyl prednisolone partially suppressed tumor angiogenesis in hamster cheek pouches under certain conditions, but tumor growth was not stopped. Many other publications have reported continued growth of tumors even in the presence of large amounts of cortisone. It has also been reported [Gross et al., Proc. Nat'l. Acad. Sci. USA 78:176 (1981)] that medroxyprogestrone, dexamethasone and to a lesser extent cortisone, inhibited tumor angiogenesis in rabbit corneas, while estradiol and testosterone were ineffective.

Aside from cortisone, certain other steroids are now known to successfully suppress angiogenesis when administered together with heparin or certain heparin fragments. The effective steroids have been referred to as "heparin-dependent" because heparin was (until now) unique in its

effect. The findings and the character of desirable angiostatic steroids has been discussed in "A New Class of Steroids Inhibits Angiogenesis in the Presence of Heparin or a Heparin Fragment", R. Crum, S. Szabo and J. Folkman, Science 230:1375 (1985); and in "Angiostatic Steroids", J. Folkman and D.E. Ingber, Annals of Surgery, 206:374 (1987) incorporated herein by reference for background purposes.

Heparin, a mucopolysaccharide, is a constitutent of various tissues, especially liver and lung, and mast cells in several mammalian species. Chemically, it has been described as an α, β glycosidically linked sulfated copolymer of D-glucosamine and D-glucuronic acid. However, although heparin has been used clinically as an anticoaggulant for half a century, both the exact structure of heparin and the precise nature by which it acts in blood anti-coaulation have not been discovered. Much of the difficulty in determining the structure of heparin results from its complexity and the fact that it is not a homogeneous, well-defined substance. Heparin is polydisperse with a molecular weight range from about 5,000 to 40,000. Within a given chain, there are also structural variations such as the varying degrees of sulfation, N-acetylation and C-5 epimerization in the uronic acid residue.

A major disadvantage in the use of heparin with a steroid to inhibit angiogenesis results from the fact that heparins manufactured by different processes and different companies revealed quite different antiangiogenic activities despite similar anticoagulant activities. The precise composition of commercial heparin apparently varies depending on its source and method of manufacture. While some heparins may be combined with cortisone to inhibit angiogenesis, other heparins are not effective as such. In fact, some heparins in order to be effective may be required in such high doses that administration may cause problems due to the anticoagulant activity of heparin. A second disadvantage is

that while heparins apparently can inhibit the growth of responsive tumors when administered in the proper dose range and proper ratio to steroid, and even , promote regression at somewhat higher doses and ratios; heparins can also cause resumption of rapid tumor growth when administered at even higher dose levels and ratios to steroid. The apparent presence of both positive and negative regulators of angiogenesis in heparin may create problems in properly administering the drug. An additional disadvantage derives from the anticoagulant activity of heparin, restricting its use to low dosage levels or to oral administration in order to avoid bleeding. Finally because heparin cannot penetrate the corneal membrane, it cannot be topically applied to the exterior of the cornea for its desired antiangiogenic activity.

2.2. CYCLODEXTRINS

Cyclodextrins (hereinafter referred to for convenience as CD or CDs for the singular and the plural, respectively) are cyclic oligosaccharides consisting of at least six glucopyranose units. Although CDs with up to twelve glucopyranose units are known, only the first three homologs have been studied extensively. These compounds have the simple, well-defined chemical structure shown in FIG. 1(A). The common designations of the lower molecular weight α-, β- and γ-CDs are used throughout this specification and will refer to the chemical structure shown in FIG. 1(A) wherein n=6, 7, or 8 glucopyranose units, respectively. The initial discovery of the CDs as degradation products of starch was made at about the turn of the century, and Schardinger showed that these compounds could be prepared by the action of Bacillus macerans amylase upon starch. In older literature, the compounds are often referred to as Schardinger dextrins. They are also sometimes called cycloamyloses.

Topographically, the CDs may be represented as a torus, as shown in FIG. 1(B), the upper rim of which is lined with primary $-CH_2OH$ groups, and the lower rim with secondary hydroxyl groups. Coaxially aligned with the torus is a channel-like cavity of about 5, 6 or 7.5 A.U. diameter for the α-, β-, and γ-CDs, respectively. These cavities make the cyclodextrins capable of forming inclusion compounds with hydrophobic guest molecules of suitable diameters.

A reasonably large number of CD derivatives have been prepared and described in the literature. In general, these chemically modified CDs are formed by reaction of the primary or secondary hydroxyl groups attached to carbons 2, 3 or 6 [FIG. 1(A)], without disturbing the α (1 --> 4) hemiacetal linkages. A review of such preparations is given in "Tetrahedron Report Number 147, Synthesis of Chemically Modified Cyclodextrins", A.P. Croft and R.A. Bartsch, Tetrahedron 39(9):1417-1474 (1983), incorporated herein by reference for background (hereinafter referred to as "Tetrahedron Report No. 147").

In particular, α-, β-, and γ-CD sulfates (Na salt) are shown as Compound Nos. 207, 208, and 209 in Tetrahedron Report No. 147, (supra) Table 26, p.1456. U.S. Patent 2,923,704 to Berger describes the preparation of cycloamylose sulfates. U.S. Patent 4,020,160 to Berstein et al. and U.S. Patent Nos. 4,247,535 and 4,258,180 to Lewis et al. disclose the use of modified cyclodextran sulfates as complement inhibitors. U.S. patent 4,383,992 to Lipari describes the preparation of a water-soluble inclusion compound of a steroid and unmodified β-cyclodextrin. U.S. patent 4,596,795 to Pitha discloses the administration (by the sublingual or buccal route) of sex hormones, particularly testosterone, progesterone and estradiol in the form of their inclusion compounds with hydroxypropyl-β-CD or poly-β-CD. None of the foregoing references are believed to show or make obvious applicants' invention as described and claimed herein.

3. ADVANTAGES AND OBJECTS OF THE INVENTION

We have now found that certain simple and very water-soluble derivatives of the cyclodextrins when administered together with a latent growth-inhibiting steroid such as cortisone or hydrocortisone or with a non-steroidal growth-inhibiting organic compound effectively inhibit angiogenesis without exhibiting the undesirable properties of heparin.

One of the objects of the present invention is to provide a novel composition including a derivative of a cyclodextrin and a growth-inhibiting compound, which composition is effective for inhibiting cell or tissue growth, especially angiogenesis, or for treating tumors, in mammals, including humans.

Another object of the invention is to provide pharmaceutical preparations containing a highly water soluble cyclodextrin derivative, especially a cyclodextrin sulfate salt, and one or more steroid compounds.

Another object of the present invention is to provide a method of treating diseases or conditions associated or characterized by angiogenesis by inhibiting undesired angiogenesis in mammals, including humans, in need of such treatment.

A further object of the present invention is to provide a method for treating mammals, including humans, having a tumor burden, to arrest and/or to regress growth of the tumor masses.

These and other objects, aspects and advantages of the present invention will become apparent to those skilled in the art upon reviewing the following description and appended claims.

4. SUMMARY OF THE INVENTION

This invention provides a composition for inhibiting undesired or pathological cell or tissue growth (including angiogenesis) in mammals, including humans, said composition

comprising active agents consisting essentially of (1) a very water-soluble derivative of α-, β- or γ-cyclodextrin in combination with (2) a latent growth-inhibiting steroid or a non-steroidal growth-inhibiting organic compound.

This invention further provides a method of inhibiting undesired or pathological cell or tissue growth (including angiogenesis) in mammals, including humans, comprising administering thereto a growth-inhibiting amount of active agents consisting essentially of (1) a very water-soluble derivative of α-, β- or γ- cyclodextrin in combination with (2) a latent growth-inhibiting steroid or a non-steroidal growth-inhibiting organic compound. This method of the invention can be accomplished either by mixing the two active agents and administering the combination via a single route or, alternatively, by administering each of the active agents separately and permitting the combination to form in vivo. According to the alternative mode, the two active agents can be administered separately via the same or different routes, so long as both agents are thus allowed to be present simultaneously in combination in vivo.

This invention further provides a method of inhibiting angiogenesis in mammals, including humans, comprising administering thereto an angiogenesis-inhibiting amount of a composition comprising active agents consisting essentially of (1) a very water-soluble derivative of α-, β- or γ-cyclodextrin in combination with (2) at least one angiogenesis inhibitor selected from the group consisting of a latent growth-inhibiting steroid and a non-steroidal growth-inhibiting organic compound, said derivative being characterized by a solubility in distilled water of at least about 20 grams per 100 milliliters of water at 0°C.

This invention further provides a method of inhibiting the pathological growth of smooth muscle cells in mammals, including humans, in need of such treatment, which method comprises administering thereto a growth-inhibiting amount of

a composition comprising as active agent a very water-soluble cyclodextrin derivative; preferably a very water-soluble cyclodextrin sulfate salt consisting essentially of the sulfated anion of α-, β- or γ-cyclodextrin associated with a non-toxic physiologically acceptable cation.

5. BRIEF DESCRIPTION OF THE FIGURES

The present invention may be more fully understood by reference to the following detailed description of the invention, examples of specific embodiments of the invention and appended figures in which:

FIG. 1(A and B) is a schematic representation of: (A) the chemical structure of α-, β- and γ- cyclodextrins; and (B) of the three-dimensional shape of these cyclodextrins.

FIG. 2 (A and B) graphically illustrates the effect of β-cyclodextrin tetradeca sulfate (β-CD-TDS) or heparin on growth of (A) rat aortic smooth muscle cells and (B) calf aortic smooth muscle cells in tissue culture.

FIG. 3(A - D) is a photographic representation of the effect of topically administered agents on endotoxin-induced capillary vessel development in the rabbit cornea. Effects of (A) Endotoxin alone (i.e., Control Group); (B) hydrocortisone alone, (Group 2); (C) β-CD-TDS + hydrocortisone, (Group 3); and (D) β-CD-TDS alone (Group 4) are shown. See text for experimental details.

FIG. 4 graphically illustrates the effect of implantation of sustained release polymers incorporating various agents on endotoxin-induced capillary blood vessel elongation in the rabbit cornea. [O] = Endotoxin alone, (Control Group); [Θ] = β-CD-TDS + Cortexolone, (Group 2); [Δ] = Cortexolone alone, (Group 3); and [□] = β-CD-TDS alone, (Group 4). See text for experimental details.

6. DETAILED DESCRIPTION OF THE INVENTION

The present inventors searched for a compound other than heparin, which would not have the disadvantages of heparin, but which when combined with a steroid would be effective for suppressing undesired or pathological cell or tissue growth including angiogenesis and, therefore, would be effective, inter alia, for controlling or eliminating tumors in mammals, including humans.

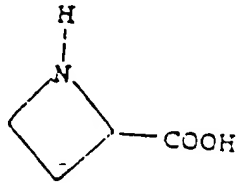
Because of the known ability of cyclodextrins including β-CD to form water soluble inclusion compounds with steroids, we attempted to make use of mixtures of unmodified CDs with hydrocortisone. However, it was discovered that such mixtures had no effect for suppressing angiogenesis, as shown by Examples 18-20 (infra, Section 7).

Quite surprisingly, we discovered that highly water-soluble salts of cyclodextrin in combination with a steroid, such as hydrocortisone, were effective for inhibiting angiogenesis. In particular, β-CD tetradeca sulfate (β-CD-TDS) was found to be very effective. In other words, a composition comprising a steroid and a water-soluble cyclodextrin derivative is effective for inhibiting undesired cell or tissue growth, including angiogenesis, and for treating tumors in mammals.

The CD sulfates and other highly water-soluble derivatives discussed herein have been found to be reproducible in their effect in the chick chorioallantoic membrane (CAM)-assay described below. This assay has been employed as a model assay to detect angiogenic activity of various substances. [Klagsbrun et al., Cancer Res. 36:10(1976)]. Three separate batches of β-CD-TDS prepared by the method described in Example 1(A) were compared in the CAM assay. Results for the three separate batches were indistinguishable. The highly water-soluble CD derivatives mimic the advantageous features of antiangiogenic heparin without its disadvantages. These findings also set aside the

notion that heparin is unique in its role in suppressing angiogenesis when combined with a suitable steroid.

We have also found that use of a highly water-soluble CD derivative such as β-CD-TDS together with a non-steroidal compound that inhibits growth in the absence of exogenous heparin, i.e., a compound that inherently has some antiangiogenic activity, surprisingly potentiates the antiangiogenic activity of such compound. Examples of such non-steroidal compounds include, but are not limited to proline analogs such as L-2-azetidinecarboxylic acid,



(see, infra, Example 24), cishydroxyproline, or 3,4-dehydroproline, and transretinoic acid. L-2-azetidinecarboxylic acid is described in Merck under Compound 911, which description is incorporated herein by reference. [See Ingber and Folkman, Lab. Investig. 59:44 (1988) for a description of such non-steroidal growth inhibiting compounds which are proline analogs.]

To clearly distinguish the steroids (which in the absnce of exogenous heparin, have no inherent antiangiogenic activity) from such non-steroidal growth-inhibitory compounds, the qualifying phrase "latent growth-inhibiting" is used herein. The adjective "non-steroidal" as used herein means a compound in which carbon ring structure characteristic of a sterol is absent.

6.1. WATER-SOLUBLE DERIVATIVES OF CYCLODEXTRINS

Highly water-soluble CD derivatives bearing non-ionic and/or ionic substituents are useful for inhibiting undesired growth according to the present invention. Suitable highly

BACKFILE DOCUMENT INDEX SHEET

A DOCPHOENIX

APPL PARTS

IMIS ____ Internal Misc. Paper

LET. ____ Misc. Incoming Letter

371P ____ PCT Papers in a 371Application

A... ____ Amendment Including Elections

ABST ____ Abstract

ADS ____ Application Data Sheet

AF/D ____ Affidavit or Exhibit Received

APPENDIX ____ Appendix

ARTIFACT ____ Artifact

BIB ____ Bib Data Sheet

CLM ____ Claim

COMPUTER ____ Computer Program Listing

CRFL ____ All CRF Papers for Backfile

DIST ____ Terminal Disclaimer Filed

DRW ____ Drawings

FOR ____ Foreign Reference

FRPR ____ Foreign Priority Papers

IDS ____ IDS Including 1449

6/11/1 NPL 5 Non-Patent Literature

OATH ____ Oath or Declaration

PET. ____ Petition

RETMAIL ____ Mail Returned by USPS

SEQLIST ____ Sequence Listing

SPEC ____ Specification

SPEC NO ____ Specification Not in English

TRNA ____ Transmittal New Application

OUTGOING

CTMS ____ Misc. Office Action

1449 ____ Signed 1449

892 ____ 892

ABN ____ Abandonment

APDEC ____ Board of Appeals Decision

APEA ____ Examiner Answer

CTAV ____ Count Advisory Action

CTEQ ____ Count Ex parte Quayle

CTFR ____ Count Final Rejection

CTNF ____ Count Non-Final

CTRS ____ Count Restriction

EXIN ____ Examiner Interview

M903 ____ DO/EO Acceptance

M905 ____ DO/EO Missing Requirement

NFDR ____ Formal Drawing Required

NOA ____ Notice of Allowance

PETDEC ____ Petition Decision

INCOMING

AP.B ____ Appeal Brief

C.AD ____ Change of Address

N/AP ____ Notice of Appeal

PA.. ____ Change in Power of Attorney

REM ____ Applicant Remarks in Amendment

XT/ ____ Extension of Time filed separate

Internal

SRNT ____ Examiner Search Notes

CLMPTO ____ PTO Prepared Complete Claim Set

ECBOX ____ Evidence Copy Box Identification

WCLM ____ Claim Worksheet

WFEE ____ Fee Worksheet

File Wrapper

FWCLM ____ File Wrapper Claim

IIFW ____ File Wrapper Issue Information

SRFW ____ File Wrapper Search Info

REV 7/02/03

Volume 291, number 1, 50–54 FEBS 10215 October 1991

ADONIS 001457939100926J

Arg–Gly–Asp constrained within cyclic pentapeptides

Strong and selective inhibitors of cell adhesion to vitronectin and laminin fragment P1

Monique Aumailley[1], Marion Gurrath[2], Gerhard Müller[2], Juan Calvete[1], Rupert Timpl[1] and Horst Kessler[2]

[1]*Max-Planck-Institut für Biochemie, D-8033 Martinsried, Germany* and [2]*Department of Organic Chemistry, Technical University Munich, D-8046 Garching, Germany*

Received 31 July 1991

Cyclic Arg–Gly–Asp–Phe–Val peptides with either D-Phe or D-Val residues were 20- to more than 100-fold better inhibitors of cell adhesion to vitronectin and/or laminin fragment P1 when compared to a linear variant or Gly–Arg–Gly–Asp–Ser. No or only little increase in inhibitory capacity was observed for fibronectin adhesion and for the binding of platelet receptor $\alpha IIb\beta 3$ to fibrinogen. NMR studies of the two most active cyclic peptides showed for both an all-*trans* conformation with a $\beta II'$ and γ turn. Subtle conformational differences, however, exist between both peptides and may contribute to selectivity of inhibition.

Conformation; Fibrinogen receptor; Integrin; NMR; Synthetic peptide

1. INTRODUCTION

The identification of the Arg–Gly–Asp (RGD) sequence as a cell adhesion site in fibronectin [1,2] was a major breakthrough in the molecular characterization of cell–matrix interactions. It was subsequently shown that several more proteins including vitronectin, von Willebrand factor, fibrinogen, thrombospondin and laminin are RGD-dependent adhesion proteins which bind to a variety of either specific or promiscuous integrin receptors [3–6]. Each residue in the tripeptide sequence appears to be crucial since conservative replacements in most cases abolish activity of synthetic peptides [2,7]. Contributions by residues adjacent to RGD are also known [8]. Yet these data do not readily explain the selectivity of RGD recognition by distinct integrins nor the possibility that RGD in a number of proteins may be non-functional. The variation of cell adhesion-inhibiting activity by restricting the conformational space of active peptide sequences, by using them in cyclic form, could lead to components with improved activity and receptor selectivity [9]. This seems feasible as shown for a cyclic synthetic RGD-peptide being a better inhibitor of vitronectin than fibronectin adhesion [8] and for several snake venom RGD-containing peptides named disintegrins [10]. In both cases conformational constraints imposed by disulfide bridges [11,12] were important for improving biological activity. In the present approach we used RGD pentapeptides in which a single D-amino acid induces defined conformational motifs and facilitates cyclization not requiring disulfide bonds. Two variants showed 20- to 100-fold higher inhibiting activity than linear GRGDS for cell adhesion to some but not all protein substrates tested. Based on 2D NMR spectroscopy and molecular dynamics (MD) simulations in solution, we also propose a distinct relationship between activity and conformation.

Correspondence address: R. Timpl, Max-Planck-Institut für Biochemie, D-8033 Martinsried, Germany. Fax: (49) (89) 8578 2422.

2. EXPERIMENTAL

Laminin fragments P1 [13] and E8 [14] were prepared from the laminin–nidogen complex obtained from the mouse Engelbreth–Holm–Swarm tumor. Human plasma fibronectin (Behringwerke, AG) and vitronectin [15] were obtained by chromatography on heparin-Sepharose. Fibrinogen from human plasma was a kind gift of Dr H. Hörmann, Martinsried. Integrin $\alpha IIb\beta 3$ (GPIIb/IIIa) was purified from human platelets [16] and biotinylated with NHS-LC-biotin following the manufacturer's (Pierce) instruction. Cyclic peptides were synthesized by the solid phase method with Fmoc protected amino acids using dicyclohexylcarbodiimide/1-hydroxybenzotriazole as coupling reagents. After release from the resin by mild acid treatment, cyclization was achieved in high dilution with diphenylphosphoryl azide at pH 8.5, followed by removal of side chain protecting groups under strong acidic conditions. The peptides were then purified by reversed phase HPLC and characterized by fast atomic bombardment mass spectroscopy. Linear GRGDS was supplied by Dr W. König, Hoechst, AG.

Conformation of cyclic peptides was studied by NMR and MD simulations [17,18]. All NMR spectra were acquired on Bruker AMX 500 and AMX 600 spectrometers and processed on a Bruker X32 computer. The ^1H assignment and the extraction of conformationally relevant parameters (coupling constants, temperature coefficients, interproton distances) were achieved by a set of 500-MHz and 600 MHz

1D and 2D NMR spectra (TOCSY, E.COSY, NOESY). For structure refinement restrained MD simulations were performed in vacuo and in solution over 150 ps. All calculations were performed on Silicon Graphics 4D/25GT, 4D/70GTB and 4D/240SX computers using the software package INSIGHT (BIOSYM) for graphical display and model building and the programs from the Groningen molecular simulation system (GROMOS) for all energy minimization and molecular dynamics simulations [19]. The NOEs were included in the potential energy function with a harmonic potential. Parameters for MD simulations in DMSO are based on unpublished work [31].

Optimal substrate coating of microtiter wells and analysis of adherent cells by a colorimetric assay have been described [20]. The human cell lines usesd were melanoma A375, mammary epithelia HBL-100 and fibrosarcoma HT 1080 [21,22]. In the inhibition assay, a cell suspension ($0.5–2 \times 10^5$ cells/ml) in Dulbecco's modified Eagle's medium was mixed with the peptide solution and immediately placed into the coated wells. Biotinylated integrin $\alpha IIb\beta 3$ was used in binding assays with immobilized ligands [23] using streptavidin–peroxidase conjugate and 5-amino-2-hydroxybenzoic acid for detection. In inhibition a fixed concentration of biotinyl-$\alpha IIb\beta 3$ (100 nM) was preincubated with increasing amounts of peptides (1 h, 25°C), prior to adding the mixture to the fibrinogen coat for another hour.

3. RESULTS AND DISCUSSION

Previous studies have shown that levels of inhibition of cell adhesion by a linear GRGDS peptide or other linear variants differ with the substrate used, being high (IC_{50} ~ 10–50 μM) for vitronectin and laminin fragment P1 but low (IC_{50} ~ 100–1000 μM) for fibronectin [7,13]. We have compared these substrates using cyclic RGDFV peptides and linear GRGDS as inhibitors. Introduction of D-Phe into the cyclic structure (cRGDFV) increased inhibition of A375 cell adhesion to laminim P1 by 20-fold and to vitronectin by 100-fold compared to GRGDS (Fig. 1). The use of D-Val in the cyclic sequence was more selective as it showed a distinct increase in inhibitory activity only for laminin P1 substrate. These observations were confirmed with 2 other cell lines and extended to more variants of the cyclic structure (Table I). Introduction of a single D-Arg or D-Asp into the cyclic peptide produced a distinct drop in inhibiting activity below the level of linear GRGDS. The same was observed for replacing Gly in the cyclic peptides by either D-Ala or L-Ala, in the latter case the D-Phe being maintained.

The increase in activity for some cyclic peptides is apparently due to cyclization rather than introducing a D-amino acid as shown for linear RGDFV which in most cases was of distinctly less inhibitory activity compared to GRGDS (Table I). Variations in this activity have been previously recognized in some, but not all, linear RGD sequences being adjacent to a hydrophobic amino acid residue [8,24].

The adhesion of 2 cell lines (A375, HBL-100) to vitronectin very likely includes in part the classical vitronectin receptor $\alpha v\beta 3$ present on these cells to a variable extent [21]. Since we could only partially inhibit adhesion by either a $\beta 3$-specific monoclonal antibody C17 [25] or an antiserum against fibronectin receptor with a strong titer for the $\beta 1$ subunit, the more recently described vitronectin receptors [6] $\alpha v\beta 1$ and possibly $\alpha v\beta 5$ may also participate. Similar antibody inhibition patterns were also observed for adhesion to laminin P1 [21] while an inhibiting monoclonal antibody to the fibronectin receptor $\alpha 5$ subunit was inactive. This indicates that high inhibiting activity of cyclic pentapeptides may involve several but not all RGD-dependent integrins.

The 3 cell lines (Table I) also adhere strongly to fibronectin and 2 of them, HT1080 and HBL-100, to collagen type IV and laminin fragment E8. These interactions were used to compare GRGDS with the most active cRGDFV peptide. Both peptides were of similar inhibiting activity (IC_{50} ~ 270–500 μM) for fibronectin adhesion demonstrating that the high activity of the cyclic D-Phe peptide (IC_{50} = 0.1–4 μM) with vitronectin and laminin P1 substrates is an effect which is specific for the receptor(s) recognizing these substrates. Cell ad-

Table I

Inhibitory capacity (IC_{50}) of cyclo (Arg–Gly–Asp–Phe–Val) derivates containing D-amino acids or Gly-to-Ala substitutions for cell adhesion on vitronectin (VN) or laminin fragment P1 and for $\alpha IIb\beta 3$ integrin binding to fibrinogen (Fb). Comparison was with linear GRGDS and RGDFV peptides

Inhibitor	IC_{50} (μM)						
	A375		HBL-100		HT1080		$\alpha IIb\beta 3$
	P1	VN	P1	VN	P1	VN	Fb
cRGDEV	2	0.2	0.1	0.1	0.2	4	12
cRGDFV	9	20	0.9	30	1.0	>120	2
cRGDFV	114	>120	25	>120	18	>120	150
cRADFV	>120	46	28	41	18	>120	>750
cRADFV	>120	>120	>120	>120	>120	>120	80
cRGDFV	>120	>120	20	>120	49	>120	nt
GRGDS	18	15	5	14	4	80	6
RGDFV	29	82	42	>170	92	>150	nt

nt = not tested.

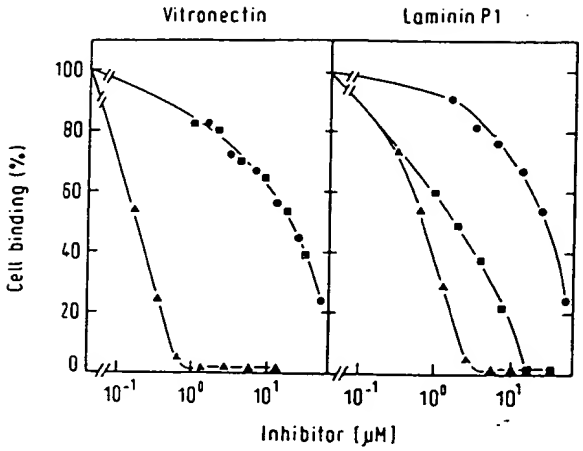


Fig. 1. Inhibition of A375 cell adhesion to vitronectin and laminin fragment P1 substrates by linear GRGDS peptide (●) or the cyclic pentapeptide RGDFV possessing either D-Phe (▲) or D-Val (■). Cell adhesion in the absence of inhibitors was set at 100%.

hesion to collagen IV and laminin E8 substrates was insensitive to inhibition by both peptides, with IC_{50} values distinctly above 500 μM. This is in accordance with previous data [21,22,26] showing that these adhesions are mediated by integrins $\alpha 1\beta 1$, $\alpha 2\beta 1$ or $\alpha 6\beta 1$ which belong to the RGD-insensitive cell receptors [6]. It also indicates that the high inhibiting activity of cRGDFV peptide for vitronectin and laminin P1 adhesion is not due to cytotoxic or other effects unrelated to integrin recognition.

Purified platelet integrin $\alpha IIb\beta 3$ was used to analyze RGD-dependent binding to fibrinogen. In the biotinylated form it bound strongly to immobilized fibrinogen but not to vitronectin and laminin P1. The binding to fibrinogen could be most efficiently inhibited by soluble fibrinogen with IC_{50} = 0.17 μM. About 10-fold higher concentrations were required for the same inhibition with the most active cyclic peptide cRGDFV (Fig. 2). Other peptides tested were of either moderately

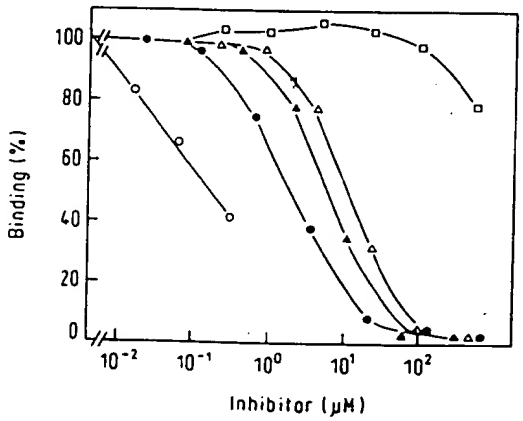


Fig. 2. Inhibition of binding of biotinylated integrin $\alpha IIb\beta 3$ to immobilized fibrinogen. Inhibitors used were fibrinogen (○), linear GRGDS (▲) and the cyclic peptides cRGDFV (●), cRGDEV (△) and cRADFV (□). Non-inhibited binding is set at 100%. Underlined residues correspond to D-isomers.

(GRGDS, cRGDEV) or distinctly lower inhibitory capacity (Table I).

The conformations of the 2 most active inhibitors cRGDEV and cRGDFV, were established by 2D NMR spectroscopy and MD simulations performed in a DMSO solvent box [17,18,27]. Both peptides were similar in adopting an all-*trans* conformation with a βII' turn and γ turn structure in which the D-amino acid occupies the i+1 position of the βII' turn (Fig. 3). The major difference is the position of the RGD sequence relative to the βII' turn and γ turn, and the formation of a different amide–carbonyl hydrogen bond stabilizing the βII' turn. These bonds are between Arg–amide and Asp–carbonyl in the D-Phe peptide and between Gly–amide and Phe–carbonyl in the D-Val peptide. Interestingly, the distance between Phe-carbonyl and Gly-amide in the D-Phe peptide is relatively short (3.1 Å) strongly suggesting that the peptide may change to the conformation of the D-Val variant. Model building studies in fact support such a transition from one to another βII'γ conformation. One can simulate by MD with dihedral restraining a conformational transition of cRGDEV via an intermediate $\beta I\gamma_i$ turn arrangement, shifted by one residue compared to the initial conformation. A conversion of the γ_i into a γ turn results in a backbone conformation identical to that determined for cRGDFV. A comparable turn rearrangement is not so easy to perform for cRGDFV, because it lacks the structural requirements for the interconversion into the conformation of cRGDEV. For example, the large distance (7 Å) between Asp–carbonyl and Arg–amide in cRGDFV prevents a similar conformational change. Hence, the proposed transition between 2 conformations could easily explain why the D-Phe peptide efficiently competes against cell adhesion to both, vitronectin and fragment P1, while the less flexible D-Val peptide may be a more selective inhibitor for laminin P1 adhesion.

The strong reduction in inhibiting activity after Gly to Ala substitution in the cyclic pentapeptide is most likely explained by steric hindrance of binding by a single methyl group such as found before for linear RAD sequences [2,7]. This is particularly obvious for cRADEV which should have a conformation almost identical to that of active cRGDEV while loss of activity in cRADFV may also be due to a shift in the positions of the βII' and γ turns. Such shifts could also explain the low activity of the peptides cRGDFV and cRGDFV which by analogy to the conformations shown in Fig. 3 would place the essential RGD sequence into a different context within the βII'γ turns. The low activity of the cyclic D-Arg variant was of particular interest, since replacement of L-Arg by D-Arg in the linear GRGDSP peptide did not cause any substantial change in inhibiting activity for vitronectin and fibronectin adhesion substrates [8].

In order to further characterize the conformational

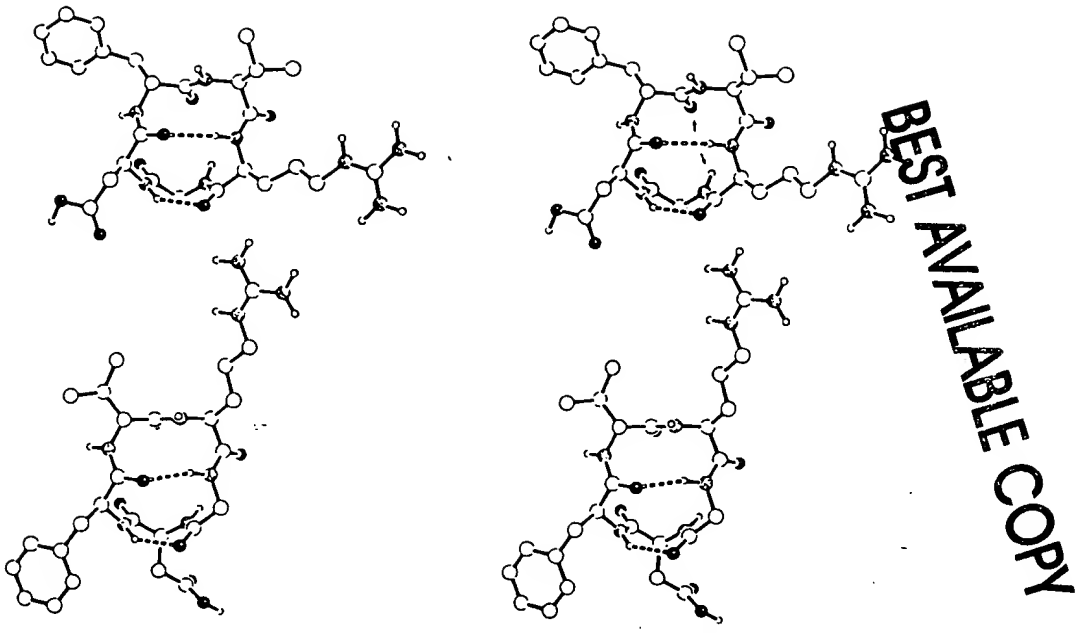


Fig. 3. Stereoplots of the conformations of the most active peptides cRGDEV (top) and cRGDFV (bottom) based on 2D NMR and MD simulation. Large circles show carbon (open), oxygen (filled) and nitrogen (stippled) atoms. Polar hydrogen atoms are indicated by small circles. The βII′ turn is shown at the top of each peptide. Essential hydrogen bonds stabilizing the βII′ turn and the γ turn are indicated by dashed lines. Arrows in the upper peptide indicate amide and carbonyl groups involved in a possible transition to the conformation of the lower peptide. Both conformations are consistent with experimental NOE data and the temperature coefficients of the NH resonances of protons involved in hydrogen bonds being in the range of $\Delta\delta/\Delta T$ = −0.75 to 3.78 ppb/K.

constraints which impose high biological activity upon the cyclic RGDEV peptide, several cyclic hexapeptides cRGDFVA containing a single D-amino acid were synthesized. Peptide cRGDEVA, which most closely resembles one of the active cyclic pentapeptides, was of 3-5-fold lower inhibiting activity for laminin P1 adhesion when compared to linear GRGDS. Conformational analysis of this hexapeptide showed, as expected, an all-*trans* conformation with D-Phe in i+1 position of a βII′ turn, but a βII turn with Arg in i+1 and Gly in i+2 positions instead of a γ turn. The distance between β-carbons of Arg and Asp which was 7.8 Å in the hexapeptide and only 6.6 Å in the pentapeptide was a further substantial difference. We assume that these conformational differences are mainly responsible for the large differences in biological activity between both cyclic peptides. We have previously also utilized a synthetic peptide for inhibition [13] comprising the authentic laminin A chain sequence [28] CQAGTFALRGDNPQGCSP-amide. This peptide, when used in either linear or cyclic disulfide-bonded form, was no better an inhibitor than GRGDS for P1 substrates. This emphasizes that the steric restrictions imposed in small rings are crucial for the specific conformation required for efficient binding to RGD-sensitive integrins. In addition, the precise position of RGD within βII′ and γ turns as well as the chirality of the spacer residues (Phe, Val) may contribute to the activity and selectivity of binding.

Other studies have shown that the disulfide-bonded peptide GPenGRGDSPCA (where Pen is penicillamine) inhibits cell adhesion to vitronectin about 10-fold better than the linear variant [8]. NMR analysis of this peptide [11] demonstrated 2 consecutive βI turns and a γ turn. This particular conformation may explain the lower relative inhibiting activity observed for this structure when compared to cRGDEV (Table I). A comparable 100-fold increase in inhibiting activity for vitronectin adhesion was recently reported for the cyclic heptapeptide cGRGDSPA when compared with the linear form, while smaller cyclic RGD peptides were far less active [29]. Here, it remains interesting to analyze to what extent the conformation of the cyclic heptapeptide comprises that of cRGDEV.

The high inhibiting activity of cyclic RGDEV and RGDFV peptides for certain but not all adhesion reactions was not predictable. However, the synthetic approach adopted was successful and also permitted the correlation of activity with distinct conformational features. The selectivity of inhibition will be useful in analyzing the receptors involved and to study their biological functions. In addition, such cyclic peptides are likely to be metabolically more stable than linear sequences, which may be an advantage for in vivo studies of developmental processes or tumor metastasis [30].

Acknowledgments: We acknowledge the expert technical assistance of Mrs Mischa Reiter and Heidi Alberty, Dr G. Schnorrenberg for providing facilities for peptide purification, Dr W. König for stimulating discussions and Drs A. Sonnenberg and G. Tarone for the gift of anti-integrin antibodies. The study was supported by grants of the Deutsche Forschungsgemeinschaft (Ti 95/7-1 and grants to HK) and the Fonds der Chemischen Industrie (HK). The support by fellowships

from Studienstiftung des deutschen Volkes (to MG). Fonds der Chemischen Industrie (to GM) and Max-Planck-Gesellschaft (to JC) is gratefully acknowledged.

REFERENCES

[1] Pierschbacher, M.D. and Ruoslahti, E. (1984) Nature 309, 30–33.
[2] Pierschbacher, M.D. and Ruoslahti, E. (1984) Proc. Natl. Acad. Sci. USA 81, 5985–5988.
[3] Ruoslahti, E. and Pierschbacher, M.D. (1987) Science 238, 491–497.
[4] Albelda, S.M. and Buck, C.A. (1990) FASEB J. 4, 2868–2880.
[5] Humphries, M.J. (1990) J. Cell Sci. 97, 585–592.
[6] Ruoslahti, E. (1991) J. Clin. Invest. 87, 1–5.
[7] Hautanen, A., Gailit, J., Mann, D.M. and Ruoslahti, E. (1989) J. Biol. Chem. 264, 1437–1442.
[8] Pierschbacher, M.D. and Ruoslahti, E. (1987) J. Biol. Chem. 262, 17924–17928.
[9] Kessler, H. (1982) Angew. Chem. 94, 509–520.
[10] Gould, R.J., Polokoff, M.A., Friedmann, P.A., Huang, T.-F., Holt, J.C., Cook, J.J. and Niewiarowski, S. (1990) Proc. Soc. Exp. Biol. Med. 195, 168–171.
[11] Siahaan, T., Lark, L.R., Pierschbacher, M.D., Ruoslahti, E. and Gierasch, L.M. (1990) In: Peptides: Chemistry, Structure, Biology (Marshall, G.R. ed) pp. 699–701, ESCOM, Leiden, The Netherlands.
[12] Calvete, J.J., Schäfer, W., Soszka, T., Lu, W., Cook, J.J., Jameson, B.A. and Niewiarowski, S. (1991) Biochemistry 30, 5225–5229.
[13] Aumailley, M., Gerl, M., Sonnenberg, A., Deutzmann, R. and Timpl, R. (1990) FEBS Letters 262, 82–86.
[14] Paulsson, M., Aumailley, M., Deutzmann, R., Timpl, R., Beck, K. and Engel, J. (1987) Eur. J. Biochem. 166, 11–19.
[15] Yatohgo, T., Izumi, M., Kashiwagi, H. and Hayashi, M. (1988) Cell Struct. Function 13, 281–292.
[16] Rivas, G.A., Calvete, J.J. and Gonzalez-Rodriguez, J. (19
Protein Purification Express (in press).
[17] Kessler, H., Haupt, A. and Will, M. (1989) In: Computer-A
Drug Design, Methods and Applications (Perun, T.J. and Pro
C.L. ed) pp. 461–484, Marcel Dekker, Inc., New York.
[18] van Gunsteren, W.F. and Berendsen, H.J.C. (1990) Ang
Chem. Int. Ed. Engl. 29, 992–1023.
[19] van Gunsteren, W.F. and Berendsen, H.J.C. (1987) Groning
Molecular Simulation (GROMOS) Library Manual, Biom
Groningen.
[20] Aumailley, M., Mann, K., von der Mark, H. and Timpl, R. (19
Exp. Cell Res. 181, 463–474.
[21] Sonnenberg, A., Linders, C.J.T., Modderman, P.W., Damh
C.H., Aumailley, M. and Timpl, R. (1990) J. Cell Biol. 11
2145–2155.
[22] Aumailley, M., Timpl, R. and Sonnenberg, A. (1990) Exp. C
Res. 188, 55–60.
[23] Aumailley, M., Wiedemann, H., Mann, K. and Timpl, R. (19
Eur. J. Biochem. 184, 241–248.
[24] Tranqui, L., Andrieux, A., Hudry-Clergeon, G., Ryckewa
J.J., Soyez, S., Chapel, A., Ginsberg, M.H., Plow, E.F. and M
guerie, G. (1988) J. Cell Biol. 108, 2519–2527.
[25] Tetteroo, P.A.T., Landsdorp, P.M., Leeksma, O.C. and von d
Borne, A.E.G. Kr. (1983) Brit. J. Haematol. 55, 509–522.
[26] Vandenberg, P., Kern, A., Ries, A., Luckenbill-Edds, L., Ma
K. and Kühn, K. (1991) J. Cell Biol. 113, 1475–1483.
[27] Lautz, J., Kessler, H., Boelens, R., Kaptein, R. and van G
steren, W.F. (1987) Int. J. Peptide Protein Res. 30, 404–414
[28] Sasaki, M., Kleinman, H.K., Huber, H., Deutzmann, R.
Yamada, Y. (1988) J. Biol. Chem. 263, 16536–16544.
[29] Kumagai, H., Tajima, M., Ueno, Y., Giga-Hama, Y. and Ohn
M. (1991) Biochem. Biophys. Res. Commun. 177, 74–82.
[30] Ruoslahti, E. and Giancotti, F.G. (1989) Cancer Cells 1, 119–12
[31] Mierke, D.F. and Kessler, H.J., J. Am. Chem. Soc. (submitte

Volume 291, nu
© 1991 Federatio
ADONIS 0014579

Ren

Expression of mR
the rat hepatoma
contrast, specific e

1. INTRODUC

Hexokinase (
catalyzes the co
phate. Therefor
tissues in which
the activity in br
[1,2]. As the act
extremely high b
cells [2,3], much
in tumor cells.

There are thre
I, II and III, and
been cloned from
human kidney [6
[8], respectively.
very high and th
with a molecular
(EC 2.7.1.2), whic
kinase and which
half the duplicat
times called type
tailed mechanism
activity of hexok
important to kno
nase isozymes is t
is responsible for
conversion.

As a first step i
mined the types
various tissue cells
muscle, heart, and

Correspondence address
ces, University of Tok
Fax: (81) (866) 33 051

Published by Elsevier S

water-soluble CD derivatives include α, β and γ CD derivatives having non-ionic substituents including but not limited to alkyl substituents such as methyl, ethyl, etc., as well as those in which a number of hydroxyl groups are replaced by other groups so as to increase the hydrophilic activity of the CD. Such groups may include, esters, ethers, thioesters, thioethers, carboxylic acids or other groups which convey hydrophilic activity by way of polar or hydrogen bonding constituents or they may include partial hydroxyl substitution that allows better hydrogen bonding involving the remaining hydroxyl groups.

The CD derivatives useful in the present invention are highly hydrophilic and therefore very water soluble. Without wishing to be bound by theory, we believe that a highly hydrophilic character is important to allow interaction with cellular surfaces. We also believe a very high water solubility of the derivative is an important factor which cooperatively interacts with the inherent complexing ability of the CD structure to provide effective inhibition of angiogenesis with an exogenous steroid, as provided by this invention. We believe that the hydrophlilic activity is roughly indicated by the affinity to water, as measured by water solubility. It is important to measure the same at 0°C since at higher temperatures the most suitable derivative have solubilities so high that meaniningful measurments are difficult.

As shown in Table III (Examples 18-22, infra, Section 7), the most soluble derivatives (measured at 0°C) show the highest antiangiogenic activity. Of the CDs, the β-CD derivatives appear to be most effective. In general, useful potency is evident at a solubility, measured at 0°C, of at least about 15 gm/100 ml in distilled water, preferably at least about 20 gm/100 ml, more preferably about 30 gm/100 ml. All solubility measurements referred to herein relate to the solubility of the substantially anhydrous derivatives, and

when these are salts, to the anhydrous sodium form. The term "very soluble" as used herein refers to a solubility of at least 15 gm/100 ml measured as described above.

It is contemplated that very water-soluble CD derivatives bearing ionic and/or non-ionic substituents may in some instances have advantageous properties, and that these are within the scope of this invention. Although highly water-soluble derivatives in general are believed useful, salt derivatives are preferred.

The phrase "salt derivative" as used herein means an ionic compound derived from a CD by reaction with a suitable reagent. The preferred salt derivatives are provided by a cyclodextrin having substituents selected from the group consisting of sulfate, phosphate, carboxylate and mixtures thereof associated with a non-toxic, physiologically acceptable cation. Many of said preferred derivatives are known compounds. (See, Tetrahedron Report Number 147, supra). But many potentially useful forms may be variants, structurally or chemically of known compounds. They also may possess several different substituents such as is the case of the cyclodextrin propoxyl sulfate of Example 1D, which we believe has not previously been reported. Some of the preferred salt forms of the derivatives are the sodium and potassium forms, since these tend to impart increased water solubility to organic anions. The salt derivatives useful herein will exibit electrolytic conductivity and osmotic properties characteristic of electrolytes and polyelectrolytes when in aqueous solution. A particularly preferred salt derivative is β-cyclodextrin tetradeca sulfate (β-CD-TDS).

The α-, β- and γ-CD sulfate salts are all usable in the presently claimed invention. β-CD sulfate salts are preferred. Various degrees of sulfation per glucose unit can be employed, such as average of one sulfate group per two glucose units of two sulfate groups per glucose unit.

Cyclodextrins having about two sulfate groups per glucose unit are preferred. Especially preferred is β-CD-TDS which has an average of two sulfate groups per glucose unit.

6.2. STEROIDS AND NON-STEROIDAL ORGANIC COMPOUNDS

Among the steroids which are effective and can be utilized in the presently claimed invention are the following:

17 alpha, 21-dihydroxy-4-pregene-3,11,20-trione and its 21-acetate (or cortisone);

11 alpha, 17,21-trihydroxypregn-4-ene-3,20-dione (or 11 alpha hydrocortisone);

11 beta, 17 alpha, 21-trihydroxypregn-4-ene-3,20-dione (or hydrocortosone);

17 alpha, 21-dihydroxypregna-4,9(11)-diene-3,20-dione;

15 alpha, 17 alpha, 21-trihydroxy-4-pregnene-3,20-dione;

16 alpha, 17 alpha, 21-trihydroxy-6 alpha-methylpregn-4-ene-3,20-dione-21-acetate-16,17 cyclic ketal of acetone;

6 alpha-fluoro-17 alpha, 21-dihydroxy-16 beta-methyl-pregna-4,9(11)-dinene-3,20-dione;

6 alpha-fluoro-18 alpha,21-dihydroxy-16 beta-methyl-pregna-4,9(11)-diene-3,20-dione-17,21-diacetate;

6 beta, 17 alpha, 21-trihydroxypregn-4-ene-3,20-dione;

17 alpha, 21-dihydroxypregn-4-ene-3,20-dione-21-acetate;

17 alpha, 21-dihydroxypregn-4-ene-3,20-dione (or Cortexolone);

9 beta, 11 beta-epoxy-17 alpha, 21-dihydroxy-2 alpha-methylpregn-4-ene-3,20-dione-21-acetate;

17 alpha, 21-dihydroxy-16 alpha-methylpregn-4-ene-3,20-dione;

9 alpha, 11 beta-dichloro-17 alpha, 21-dihydroxypregn-4-ene-3,20-dione-21-acetate

17 alpha, 21-dihydroxy-6 alpha, 16 alpha-dimethylpregn-4-ene-3,20-dione-21-acetate;

17 alpha, 21-dihydroxy-16 alpha-methylpregna-4,9(11)-diene-3,20-dione-21-acetate;

17 alpha, 21-dihydroxy-16 beta-methylpregna-4,9(11)-diene-3,20-dione-21-benzoate;
6 alpha-fluoro-17 alpha, 21-dihydroxy-16 beta-methylpregna-4,9(11)-diene-3,20-dione-17-acetate-21-benzoate;
17 alpha, 21-dihydroxy-16 beta-methylpregna-1,4,9(11)-triene-3,20-dione-17-succinate sodium monohydrate;
9 alpha-fluoro-11 beta, 16 alpha, 17 alpha, 21-tetrahydroxy-pregn-4-ene-3,20-dione-16,21-diacetate;
17 alpha, 21-dihydroxy-16 alpha-methylpregna-1,4,9(11)-triene-3,20-dione-21-succinate sodium monohydrate;
6 alpha-fluoro-17 alpha, 21-dihydroxy-16 beta-methyl-pregna-1,4,9(11)-triene-3,20-dione-21-succinate sodium;
desoxycorticosterone;
testosterone;
estrone; and
tetrahydro S.

More preferred are those steroids which lack glucocorticoid and mineralo-corticoid activity, since such activity is an undesired effect and limits the dose size or extent of use of the steroid for the purpose of the present invention. Among such more preferred steroids are 11 alpha, 17,21-trihydroxypregn-4-ene-3,20-dione (or 11 alpha-hydrocortisone), 17 alpha, 21-dihydroxypregn-4-ene-3,20-dione (11-desoxycortisol or Cortexolone), and 17 alpha, 21-dihydroxypregna-4,9(11)-diene-3,20-dione.

None of the steroids themselves effectively inhibits angiogenesis nor causes regression of tumors in the absence of a water-soluble cyclodextrin derivative of the present invention.

As taught by the present invention, the growth-inhibitory activity of non-steroidal organic compounds is potentiated by combination with a water-soluble cyclodextrin derivative. Among the non-steroidal growth-inhibiting organic compounds which are effective and can be utilized in the presently claimed invention are the following: proline

analogs such as L-2 azetidinecarboxylic, cishydroxyproline, and 3,4-dehydroproline and transretinoic acid acid.

Additionally, any non-steroidal organic compound which in combination with a cyclodextrin derivative demonstrates growth inhibiting activity in either of the bioassays described below can be utilized in the methods of the presently claimed invention.

Several bioassays have been developed to estimate the angiogenic-inhibiting potency, if any, of a substance. The rabbit cornea is the basis of one of these methods. The cornea is avascular. A small pocket can be made in it and a tumor implant can be inserted while the rabbit is anesthetized. The tumor is separated from the vascular bed of the host. New capillary blood vessels will grow in a linear manner toward the tumor, and the rate of vessel growth can be measured. [For a more detailed description of this assay, see. Gimbrone et al., J. Nat'l Cancer Inst. 52:413 (1973) incorporated herein by reference].

A more economic bioassay makes use of the choricallantoic membrane of the chick embryo. This test will for convenience be referred to hereinafter as the "CAM assay". For a more detailed description of the CAM assay, see Folkman et al., Science 221:719 (1983), incorporated herein by reference. A typical CAM assay, such as used for the evaluations in the examples in Section 7, infra, employs 16 eggs per experiment. A 2 mm diameter disk of methylcellulose containing the test substance is applied to the chorioallantoic membrane of a 6-day chick embryo, cultured in a Petri dish, in a humidified incubator with 3% carbon dioxide. Two days later (8-day embryo), the membrane is examined under a stereomicroscope at six- to ten-fold magnification. Inhibition of angiogenesis by the test substance is evidenced by the development of an avascular zone around the methylcellulose disc. An avascular zone of 4 mm is graded as (++) and an avascular zone of 2 mm is graded

at (+). The potency of the inhibition at the 2 mm and 4 mm zone(s) are expressed as the percentage of the total number of eggs (usually 16) in the test that were rated (++) or (+), i.e., the % of "successes". A rating of zero % reflects absence of inhibition of the test substance under the test conditions.

The sustained release methylcellulose discs are prepared by dispersing appropriate amount(s) of the test substance of substances in an 0.45% aqueous solution of methylcellulose, and depositing 10 microliter aliquots of the resulting solution in a Teflon mold, followed by air drying for about one hour in a laminar flow hood.

A very advantageous feature of the CAM assay is the very high sensitivity of the chick embryo to toxic substances. Moreover, the lack of toxicity of a substance in the CAM assay has been correlated with lack of toxicity of such substance when administered to other animals.

6.3. APPLICATIONS AND METHODS OF USE

The composition of the present invention is useful for inhibiting undesired cell and tissue growth, including angiogenesis. Of course, the composition of the present invention comprising a water soluble derivative of an α-, β- or γ-CD and a steroid is to be administered to mammals including humans in need of such treatment. For example, mammals with tumors are in need of treatment with the composition of the present invention. While not completely understood, it is believed that treatment with the composition of the present invention inhibits the creation of new capillaries necessary for tumor growth. This results in the tumor having an insufficient supply of nutrients essential for its growth or even for its vitality. Thus, tumors in mammals including humans, when treated in accordance with the present invention, do not grow and may even lose their vitality and die. Among the tumors

contemplated as responsive to the composition and methods of this invention are Reticulum Cell Sarcoma, Lewis Lung Carcinoma, B-16 Melanoma, and Bladder Carcinoma, etc.

Neither mature non-growing blood vessels nor vascular tissue appear to be affected by the treatment with the composition of the present invention. Inhibition of angiogenesis in accordance with the present invention, in addition to its effect upon tumor regression and metastasis in tumor-bearing animals, may be effective in treating a number of other ailments.

The present invention further provides a method for treatment of a number of other non-tumorous disorders which are characterized by pathological cell or tissue growth, including angiogenesis. Thus the invention provides a method for treatment of mammals, including humans, afflicted with a number of non-neoplastic pathological conditions including rheumatoid arthritis, in which abnormal capillary growth can destroy joint cartilage; hemanogiomas, in which abnormal capillary proliferation appears in newborns and can persist for up to 2 years; angiofibromas which develop in the nasopharynx; psoriasis, in which excessive proliferation and shedding may be dependent on abnormal capillary growth in the dermis. Additionally, the present invention provides a method for treatment of a number of ophthalmological pathologies which are associated with undesired angiogenesis, including diabetic retinopathy, retrolental fibroplasia and neovascular glaucoma.

The present invention further provides a method for inhibiting undesired smooth muscle cell development often observed following angioplasty or treatment to remove atherosclerotic plaques which occlude blood vessels.

According to one embodiment of the method of the present invention, the active agents are mixed together prior to administration so that the steroid compound or non-steroidal growth-inhibiting compound is administered in

combination with the water-soluble cyclodextrin derivative. After the mixture is prepared, it may be administered orally or parenterally including, inter alia, topical application, intravenous, intra-arterial or subcutaneous injection, and including absorption as well as injection and introduction into body apertures or orifices.

Cortisone and its physiclogically accepted non-toxic derivatives, such as the acetates, as well as many other steroids useful in the present invention, are only slightly soluble in water. However, when combined with the water-soluble cyclodextrin derivatives of the invention, the resulting complexes have increased water solubility. Accordingly, the composition of the present invention can easily be administered. The term "cortisone" and "hydrocortisone" and 11-α isomer of hydrocortisone as used in the present specification and claims are intended to include both the steroids themselves and their derivatives and structural variants.

According to an alternate embodiment of the method of the invention, the active agents are each administered separately and the combination of the two agents forms in vivo. In this embodiment, the two active agents can be introduced separately either via the same or different routes of administration, so long as both agents are thus present simultaneously in vivo, permitting a complex mixture of the two active agents to form.

Dosages employed are limited only by the well-known limits of the administration of drugs individually for their usual effects, in the case of cortisone, hydrocortisone, or 11-α isomer. Since the CD derivatives useful herein have no anticoagulant effect and show no toxicity in the CAM test at dosages employed according to the method of the invention (see below), they may be administered percutaneously in dosages at least as large as those acceptable for heparin. Oral dosage may be considerably higher. Simple testing, for

example by the procedure of Example 3 in U.S. Patent Application Serial Number 641,305, filed August 16, 1984, suffices to determine effectiveness and optimum dose. The procedure of Example 3 is incorporated herein by reference.

The dose amount required to bring about arrest of tumor growth or regression of tumors varies depending upon the identity of the tumor, as does the length of time required to bring about arrest or regression of tumors. Tumor size at the beginning of treatment also affects the length of time for complete regression. Because administration of cortisone, with or without β-CD-TDS(Na), for example, may result in pulmonary infection after a number of days, it may be desirable to administer a suitable antibiotic as prophylaxis during treatment in accordance with the present invention. Such antibiotics can be mixed with the water-soluble cyclodextrin derivative and the steroid or non-steroidal growth-inhibiting agents of the invention and administered as a mixture or, alternatively, the antibiotics can be administered alone contemporaneously with the water-soluble cyclodextrins and growth-inhibiting agents of the invention either by the same or a different route of administration.

As shown in Table I, infra Section 7, it appears that a weight ratio greater than about 2:1 or a molar ratio of greater than about 0.4 of water-soluble CD derivative to steroid leads to a decrease of antiangiogenic activity. The preferred molar range and the molar ratio of CD derivative to angiostatic agent (steroidal or non-steroidal) is 0.004 to about 0.7. A more preferred useful range is about 0.04 to about 0.7. The latter range is particularly useful in topical applications, including as eye drops for ophtalamological uses. The amount of angiostat will generally be of the order of 0.1 to 10 mg/ml when admixed with the CD derivative, however, the absolute amount may depend on the mode and frequency of administration

The effective compositions of this invention are best administered in a suitable carrier, which must be non-toxic and physiologically acceptable, such as water or normal saline solution. Compositions containing mixtures of the active agents or each of the active agents alone, either dry or in a suitable carrier, can be employed.

7. EXAMPLES

The following examples are provided to illustrate this invention. However, they are not to be construed as limiting the scope of the invention, which scope is determined by this entire specification including the appended claims. All amounts and proportions shown are by weight unless explicitely stated to be otherwise. For the CAM assay, however, % refers to the number of "successes". (See Section 6.3, above).

Examples 1 (A-D)

This example illustrates the best mode known to us for preparing and purifying cyclodextrin sulfates. The method is not per se considered part of the present invention.

(A) β CD-TDS(Na):

β-cyclodextrin (99% pure dihydrate) was purchased from Chemalog (a division of General Dynamics Corp.), South Plainfield, New Jersey.

5.0 grams of β-cyclodextrin (4.4 mmoles, i.e., about 92 meq -OH) was dissolved in 250 ml of dimethylformamide (DMF). To this solution was added 15.0 grams of $(CH_3)_3N{\cdot}SO_3$ (108 mmoles) in a single portion and the reaction mixture was heated to 70°C. After two hours at 70°C, a gummy material began to precipitate. The reaction mixture was maintained at 70°C with vigorous stirring, and then cooled to room temperature. The DMF layer was then decanted and discarded, and the solid residue was dissolved in 250 ml of water

followed by addition of 75 ml of 30% sodium acetate. The mixture was stirred vigorously for 4 hours and then poured into 4000 ml of ethanol. After standing overnight, the mixture was filtered to recover the crystallized solids. The filter cake was washed with ethanol (absolute) followed by diethyl ether. The product was then dried under vacuum over P_2O_5. 10.3 grams of white powder was recovered. The product was hygroscopic.

The product was analyzed under conditions such that sorption of water was minimized. Elemental analysis gave the following results: C=18.84, H=2.65, S=17.33 (Calculated for $C_6H_8O_{11}S_2Na_2$; C=19.67, H=2.19, S=17.49). $[\alpha]^{22}_D$=75° (C=2.63 in 0.5 M NaCl). The analysis corresponds to that expected for an average substitution of two hydroxyl groups for each glucopyranose unit, i.e., 14 hydroxyls per CD molecules. The calculated yield for such β-CD-TDS salt is 10.96 grams, about 6% higher than the observed 10.3 grams.

(B) α- and γ-CD-S (Na salt):

The procedure described above was used for these preparations except that 86 mmoles of CH_3N-SO_3 was used with α-CD, and 117 mmoles with the γ-CD.

The sulfated α-CD salt analyzed C=18.76; H=2.60; S=16.22. This corresponds on average to a substitution of about 11.7 hydroxyl units per α-CD molecule.

The sulfated γ-CD salt analyzed C=18.92; H=2.69; S=14.84. This corresponds on average to a substitution of about 14 hydroxyl groups per γ-CD molecule.

(C) β CD-SO_4 (Na salt) (7.1 wt% S):

1.0 gm of β-cyclodextrin was dissolved into 50 ml of DMF. To this solution was added 883 mg of $(CH_3)_3N{\cdot}SO_3$ (7.2 equivalents). The solution was held at 75°C for 12 hours, at which time no precipitate had formed. The reaction mixture was cooled to room temperature. To the solution was added

200 ml of ethanol. The resulting colloidal solution was then poured into 600 ml of diethyl ether. A white solid formed in 2 hours. The solid was collected by filtration and then was dissolved in 30 ml H_2O. This solution was stirred for 2 hours. After stirring, the solution was poured into 900 ml of a 2:1 EtOH-Et_2O solution. Crystals formed over an 8 hour period. The crystals were collected and washed with Et_2O. The product was dried over P_2O_5 under vacuum. 1.18 gm of powder was recovered. (72.4% yield).

Elemental analysis of the product showed C=32.49; H=4.99; and S=7.06. This corresponds on average to a substitution of about 3.5 hydroxyls per β-CD molecule.

(D) β-CD-Propoxylate -14 SO_4

β-CD-(hydroxy-n-propyl ether) was obtained from American Maize-Products Co. (Hammond, IN) and the procedure described above was used to prepare the sulfate salt, β-CD-(-4Pr-14 SO_4).

Examples 2-15

In these examples the angiogenesis-inhibiting potency of hydrocortisone with various dosage levels of β-CD-TDS prepared as in Example 1 was evaluated by the CAM assay. The methylcellulose discs all contained 60 μg of the steroid but the β-CD-TDS was varied from 100 μg down to 0.05 μg. The results are summarized in Table I. As can be seen from the data, angiogenesis inhibition persists with extremely low levels (0.05 μg) of the CD compound with no evidence of activation of angiogenesis at two thousand-fold higher concentration.

TABLE I

Example No.	Hydrocortisone µg	Beta-CD-TDS µg	CAM Assay (++) %	CAM Assay (+) %
2	60	100	—	57
3	"	50	60	100
4	"	50	22	55
5	"	25	10	60
6	"	25	18	55
7	"	10	40	70
8	"	10	6	40
9	"	5	0	50
10	"	1	0	50
11	"	1	0	42
12	"	0.5	0	40
13	"	0.1	0	45
14	"	0.1	0	37
15	"	0.05	0	20

In contrast with these results, CAM tests made with 100 µg of α-, β- or γ- cyclodextrin with 50 µg of hydrocortisone all showed total absence of angiogenesis-inhibition [no successes at either the 1 mm zone (+) or the 2 mm zone (++) level].

Examples 16-17

Examples 16 and 17 illustrate the low, but useful, activity afforded by the sulfated α-CD and γ-CD as shown in Table II. Date for Examples 5 and 6 are included for comparison. All tests were made with 25 µg of the indicated CD sulfate and 50 µg of cortisone.

TABLE II

Example No.	CD	Sulfur, Wt%	CAM Assay (+) %	CAM Assay (++) %
16	Alpha	16.2	8	0
17	Gamma	18.9	15	0
5	Beta-CD-TDS	17.3	60	15
6	Beta-CD-TDS	17.3	55	18

Examples 18-22

This group of examples shows that the angiogenesis suppression activity requires, aside from the characteristic complexing activity of the CD structure, a high water solubility. The CAM assays were made with a dosage of 50 μg to 60 μg of hydrocortisone in 10 μl of 0.45% methylcellulose in water.

In order to make comparisons that included the very water-soluble CD sulfates, the solubilities of which were so high at room temperature that measurement was not practical, all solubility measurements were made in liquid water at zero °C. One can picture this to be a measure of the competition of the hydrophile bonding with water in relation to the orderly bonding of water to itself. These comparisons are summarized in Table III.

Examples 18, 19 and 20 describe the results for the unsubstituted CDs, which gave no indication of angiogenesis suppression activity in the CAM assay. Example 21 shows the results for a sample of propoxylated β-CD (hydroxy-n-propyl ether) obtained from American Maize-Products Co. (Hammond, IN), reported to have an average of about 4 hydroxypropyl groups per CD molecule. Examples 22 shows the results obtained with the less sulfated β-CD product from Example 1(C) above. The results for Examples, 16, 17 and 5,

including water solubilities at zero °C, are included to complete the comparison.

TABLE III

CAM Assays

Ex. No.	Compound	Conc. µg/10 µl	No. Examples	% Avascular zones	Solubility, 0°C, gm/100 ml H_2O
18	α-CD	100	37	5± 0.3	6
		25	20	0	6
19	β-CD	100	19	0	0.7
		25	23	0.04	0.7
20	γ-CD	100		0	*
21	β-CD-propoxylated (~ 4 Pr)	100	52	29±10.5	20
		25	50	31± 9.7	20
21a	β-CD-14Me (~ 14 Meth)	100	57	22± 5.7	32+
		25	37	20± 4.2	32+
22	β-CD-(~ 7 SO_4)	100	25	20± 9.6	13
		25	27	8± 3.1	13
22a	β-CD (~ 4 propoxylated) (~ 14 SO_4)	25		37	39
16	α-CD	100	40	17± 4.7	36
	(~ 12 SO_4)	25	25	4± 2.7	36
17	γ-CD	100	19	32± 5.3	38
	(~ 16 SO_4)	25	20	19± 1.7	38
5	β-CD-TDS	100	101	55± 7.5	42
		50	75	75± 5.8	42
		25	107	58± 7.0	42

* Insufficient material available for solubility determination.

Example 23

This example illustrates that Cortexolone with β-CD-TDS is an exceptionally effective antiangiogenic composition. Cortexolone is closely related to cortisone chemically. However, it possesses almost none of the functions of cortisone, except for the antiangiogenic effect in the presence of heparin.

Cortexolone, 50 µl/10 µl alone, gave zero % avascular zones in the CAM assay.

At the same dosage level, with 25 µg/10 µl of β-CD-TDS, the CAM assay showed 85% avascular zones of which 31% are (++) or greater.

Example 24

This example demonstrates that the angiostatic activity of L-2-azetidinecarboxylic acid, which has some inherent angiostatic activity, is strongly potentiated by the presence of CD-TDS. The results of the CAM assay, in the absence and presence of CD-TDS, are given in Table IV, Examples 24(a) and 24(b), respectively.

TABLE IV: L-2-AZETIDINE

	μg/10 μl	μg CD-TDS	% Avascular Zones
(a) L-2-Azetidine	400	0	28
(b) L-2-Azetidine	400	25	100*

* 50% of these avascular zones were 2+, and 25% were 3+, i.e., the largest avascular zones ever observed, greater than 10 mm.

Example 25

This example demonstrates the β-CD-TDS is about three times as effective as whole heparin in suppressing smooth muscle cell (SMC) growth when each is used alone (i.e., without exogenous corticosteroid or other supplementation). The bioassay of this activity was made using tissue cultures of rat aortic SMC and calf aortic SMC, with dosages ranging from 0.03 μg/ml up to 400 μg/ml.

The results are shown graphically in FIG. 2(A) and (B).

Example 26

This example demonstrates that topical administration of β-C-TDS in combination with hydrocortisone effectively inhibits neovascularization in the cornea.

The rabbit corneal test described by Perlin, Fed. Proc. 36:101 (1977) modified as described below, was employed to examine the effectiveness of a combination of β-CD-TDS and hydrocortisone. In this corneal test, a sustained release polymer impregnated with bacterial endotoxin was first implanted in rabbit corneas. Endotoxin slowly released from this implant induces angiogenesis in the cornea in a manner analogous to the neovascularization observed in patients rejecting a corneal transplant. Rather than employing a

second implant containing the test substance to deliver such test substance as generally used, in the present experiments the test substance was applied topically to the cornea, i.e., in the form of eye drops. Animals having received an endotoxin-containing implant were divided into four groups and were treated by topical application (eye drops) as follows:

Group 1, was not treated further and served as the control group; Group 2, received hydrocortisone alone (0.5 mg/ml); Group 3, hydrocortisone-21-phosphate and β-CD-TDS, (0.5 mg/ml and 1.0 mg/ml, respectively); and Group 4, β-CD-TDS (1.0 mg/ml). The diluent for the eye drops was saline in all cases. FIG. 3 (A-D) illustrates the results typically obtained on the 9th day post-implantation and treatment.

As shown in the photographs of FIG. 3, a combination of hydrocortisone and β-CD-TDS when topically applied to the cornea was very effective in inhibiting angiogenesis (FIG. 3C). The efficacy of this treatment is particularly apparent when the results obtained are compared to those observed in the untreated or control group (FIG. 3C vs FIG. 3A). In fact, in animals treated with hydrocortisone and β-CD-TDS, capillary growth was not only inhibited, but also new capillaries which had formed before initiation of the treatment regressed (FIG. 3C). On the other hand, hydrocortisone alone produced only a slight inhibition of angiogenesis (FIG. 3B). β-CD-TDS alone caused a slight stimulation of angiogenesis (FIG. 3D).

In another series of experiments, the antiangiogenic activity of a water-soluble cyclodextrin salt derivative in combination with cortexolone was evaluated using the rabbit cornea test as described by Gimbrone et al., J. Nat'l Cancer Inst. 52:413 (1974). In these experiments, E. coli endotoxin (17 $\mu g/mm^3$) incorporated into sustained release polymer pellets of ethylene vinylacetate copolymer [Elvax, Sigma Chemical, St. Louis, MO (hereinafter "Elvax")] was implanted

between the vascular limbal edge of the rabbit corneas. The test substance(s) were administered by means of a second Elvax implant incorporating the particular test substance.

Elvax containing endotoxin was implanted into the corneas of 12 rabbits. The animals were then divided into 4 groups and 4 eyes were treated for each group as follows: Group 1, received no further treatment and served as the control group; Group 2, β-CD-TDS at 15 $\mu g/mm^3$ Elvax pellet; Group 3, cortexolone at 30 $\mu g/mm^3$ Elvax pellet; and Group 4, a combination of β-CD-TDS and cortexolone incorporated into an Elvax pellat at a final concentration of 15 $\mu g/mm^3$ Elvax of β-CD-TDS and 30 $\mu g/mm^3$ Elvax of cortexolone. The vessel length of new capillary growth was measured every 2 days with a slit-lamp stereoscope at 10X using an ocular grid calibrated to $\pm$ 0.1 mm.

Measurement of vessel length alone underestimates the extent of antiangiogenic activity because such measurement does not assess capillary density. Thus, the vessel density was also evaluated and graded using the following scale: 0=no vessels/cornea; 1=1-4 vessels/cornea; 2=5-20 vessels/cornea; 3=20-50 vessels/cornea; and 4=more than 50 vessels/cornea. This grade was then multiplied by mean maximal length of the vessels to obtain a semi-quantitative estimate of vessel density (length-density index) for each cornea. Results obtained are graphically illustrated in FIG. 4 and tabulated in Table V.

As demonstrated in FIG. 4, the largest difference between treated and control corneas was observed on day 13 after implantation of the Elvax pellets. The mean vessel lengths and vessel density observed on day 13 are listed in Table V.

TABLE V

INHIBITION OF ANGIOGENESIS IN CORNEAS

	Inhibition (% of Untreated Control Group		
	β-CD-TDS Cortexolone	Cortexolone Alone	β-CD-TDS Alone[a]
Vessel Length	18%	49%	164%
Vessel Density	8%	61%	303%

[a] Percentage greater than 100% represents stimulation of vessel development.

As shown in Table V, a combination of β-CD-TDS and cortexolone inhibited linear capillary blood vessel growth to about 18% of that observed in untreated eyes. When capillary density was estimated, this combination suppressed vessel density to about 8% of that observed in untreated eyes. In contrast as shown in Table V, when administered alone, cortexolone inhibited linear vessel growth only to about 49% and vessel density to about 61% that observed in untreated eyes. Surprisingly, as further demonstrated in Table V, administration of β-CD-TDS alone, stimulated vessel growth by about 164% and vessel density by about 303% above that observed in untreated eyes.

Based on these results, it is clear that administration of a cyclodextran salt derivative in combination with a steroid according to the present invention, is an effective method for inhibiting angiogenesis in ophthalmological tissues.

WHAT IS CLAIMED IS:

1. A composition for inhibiting undesired or pathological cell or tissue growth, including angiogenesis, in mammals, including humans, comprising (1) a derivative of α-, β- or γ-cyclodextrin in combination with (2) a latent growth-inhibiting steroid or a non-steroidal growth-inhibiting organic compound, in which the derivative is characterized by a solubility at 0°C in distilled water at least about 20 gm/ml of water.

2. The composition according to claim 1, in which the derivative of α-, β- or γ-cyclodextrin is an anionic salt derivative of said cyclodextrin having substituents selected from the group consisting of sulfate, phosphate, carboxylate and mixtures thereof asscciated with a physiologically acceptable cation.

3. The composition according to claim 2, in which the derivative of α-, β- or γ-cyclodextrin is a cyclodextrin sulfate.

4. The composition according to claim 3, in which the cyclodextrin sulfate is β-cyclodextrin tetradeca sulfate.

5. The composition according to claim 1, in which the steroid has 17-alpha and 21-hydroxyl groups, 3- and 20-one groups and in the 16-position hydrogen, hydroxy or a methyl group and non-toxic, physiologically acceptable carboxylates, acetal, ketals and phosphates thereof.

6. The composition according to claim 3, in which the steroid has 17-alpha and 21-hydroxyl groups, 3- and 20-one groups and in the 16-position hydrogen, hydroxy or a methyl

group and non-toxic, physiolcgically acceptable carboxylates, acetal, ketals and phosphates thereof.

7. The composition according to claim 4, in which the steroid has 17-alpha and 21-hydroxyl groups, 3- and 20-one groups and in the 16-position hydrogen, hydroxy or a methyl group and non-toxic, physiolcgically acceptable carboxylates, acetal, ketals and phosphates thereof.

8. The composition according to claim 7, in which the steroid is cortisone, hydrocortisone or cortexolone.

9. The composition according to claim 1, in which the non-steroidal growth-inhibiting organic compound is L-2-azetidinecarboxylic acid and the derivative of cyclodextrin is a cyclodextrin sulfate.

10. A method for inhibiting undesired or pathological cell or tissue growth, including angiogenesis, in mammals, including humans, comprising administering to a mammal a growth-inhibiting amount of active agents consisting essentially of (1) a derivative of α-, β- or γ-cyclodextrin in combination with (2) a latent growth-inhibiting steroid or a non-steroidal growth-inhibiting organic compound, the derivative being characterized by a solubility at 0°C in distilled water of at least about 20 gm/100 ml of water.

11. A method for inhibiting angiogenesis in mammals, including humans, comprising administering to a mammal an angiogenesis-inhibiting amount of active agents consisting essentially of (1) a derivative of α-, β- or γ-cyclodextrin in combination with (2) a latent growth-inhibiting steroid or a non-steroidal growth-inhibiting organic compound, the derivative being characterized by a solubility at 0°C in distilled water of at least about 20 gm/100 ml of water.

12. The method acording to claim 10, in which the derivative of α-, β- or γ-cyclodextrin is a salt consisting essentially of an anionic derivative of a cyclodextrin having substituents selected from the group consisting of sulfate, phosphate, carboxylate and mixtures thereof assocated with a non-toxic physiologically accetable cation.

13. The method according to claim 12, in which the salt of α-, β- or γ-cyclodextrin is a cyclodextrin sulfate.

14. The method according to claim 13, in which the salt is β-cyclodextrin sulfate.

15. The method according to claim 10, in which the steroid has 17-alpha and 21-hydroxyl groups, 3- and 20-one groups and in the 16-position hydrogen, hydroxy or a methyl group and non-toxic, physiologically acceptable carboxylates, acetal, ketals and phosphates thereof.

16. The method acording to claim 10, in which the steroid is cortisone, hydrocortisone or cortexolone.

17. The method according to claim 10, in which the non stercidal growth-inhibiting organic compound is L-2-azetidinecarboxylic acid.

18. A method for inhibiting the pathological growth of smooth muscle cells in mammals, including humans, comprising administering to a mammal, a growth-inhibiting amount of a derivative of α-, β- or γ-cyclodextrin, the derivative being characterized by a solubility at 0°C in distilled water of at least about 20 gm/100 ml of water.

19. The method according to claim 18, further comprising administering an amount of a latent growth-